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PRIMER NOTE

Polymorphic microsatellite markers for stock identification in Japanese anchovy (*Engraulis japonica*)

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Abstract

Japanese anchovy (*Engraulis japonica*) is a migratory marine fish of high economic significance in Taiwan. The adult Japanese anchovies migrate from the East China sea to spawn in coastal waters of Taiwan; the larvae then drift back to the East China Sea to complete their life cycle. We developed six highly polymorphic microsatellites for *E. Japonica* (expected heterozygosity ranging from 0.751 to 0.971) and these microsatellites can be used as genetic markers for identifying stocks to establish regulations in fishing management. Moreover, the markers will be useful in inferring the stock origins and migration routes in the future.

Keywords: Engraulidae, *Engraulis japonica*, microsatellite, migration, stock

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Japanese anchovy (*Engraulis japonica*) is a migratory marine fish and a key link in the food chain since their larvae feed on copepods (Hirakawa *et al*. 1997) like the European anchovy (*Engraulis encrasicolus*) (Plounevez & Champalbert 1999). Larvae of the Japanese anchovy comprise the major catch of anchovy larvae fishery, which has been a traditional fishing practice in Taiwan (Young & Chiu 1994; Chiu *et al*. 1997). The economic significance has propelled the government to embark on population studies on the species, and since 1999 have imposed a regulation of seasonal closure to harvest the anchovy larvae.

The adult Japanese anchovies migrate from the East China Sea to spawn in coastal waters of Taiwan, and the larvae then drift back to the East China Sea to complete their life cycle. We developed microsatellite primers for *E. japonica* in an attempt to identify genetic stocks of the species and to offer insights to fine-tune the regulations and measures to protect the species. Moreover, by systematic sampling of the fish from its distribution range, the markers will be useful in inferring the stock origins and migration routes in the future.

Genomic DNA for constructing the partial libraries was prepared according to procedures in Sambrook *et al*. (1989). Genomic DNA was digested with *Sau*3AI and fractioned in a 2.5% NuSieve™ GTG gel (FMC). DNA of size range of 300–900 bp was isolated, purified with GeneClean III kit (Bio101, Inc.) and ligated into plasmids, PUC18/*Bam*HI/BAP (Pharmacia) according to the manufacturers' protocols. Ligated plasmids were transformed into competent SURE cells (Stratagene). Recombinant clones containing inserts were transferred to Hybond-N^+ nylon membranes (Amersham), which were hybridized to a set of oligonucleotide probes, including $(AC)_{10}$, $(TC)_{10}$, $(CAC)_5CA$, $CT(ATCT)_6$, $(TGTA)_6TG$, and $CT(CCT)_5$. Probes were labelled with DIG Oligonucleotide 3'-End Labelling Kit (Boehringer Mannheim). Hybridization was performed at 60 °C for 24 h in a standard hybridization buffer, consisting of 5× SSC, 0.1% N-lauroylsarcosine, 0.2% SDS, and 1% Blocking Reagent (Boehringer Mannheim). The membranes were washed twice, each for 5 min at 45 °C with a solution of 2× SSC, 0.1% SDS, and then twice, each for 15 min at 65 °C with a solution of 0.1× SSC, 0.1% SDS. Chemiluminescent detection was performed with DIG Luminescent Detection Kit (Boehringer Mannheim). Exposure time ranged from 20 to 30 min. A total of 46 positive clones was sequenced on an automated sequencer (ABI/Perkin Elmer), in which 31 contained dinucleotide, trinucleotide, or other types of repeats. The online program PRIMER 3.0 (http://www.genome.wi.mit.edu) was used to design primers from flanking regions of microsatellite DNA loci that contain more than 10 repeat units except one locus of pentanucleotide repeat motif. The pentanucleotide locus presumably offers better resolution in discerning alleles.

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Table 1 Characterization of six polymorphic microsatellite loci in the Japanese anchovy *Engraulis japonica*

Locus	Repeat motif	Primer sequences	T_a (°C)	Sample size	Size range (bp)	No. of alleles	H_O	H_E	GenBank access no.
EJ2	$(CT)_{43}$	F5′ AGCAAGGGAGCAAACAATC R5′ TGCAATTTGACAGAAACCACA	58	162	149–279	53	0.438	0.966	AF344655
EJ9	$(TC)_{39}$	F5′ GCCTTACCCCTTTAGCCATT R5′ GCCCTCCGAGTCGACATAGT	60	137	220–320	45	0.540	0.957	AF344656
EJ27.1	$(GA)_{36}$	F5′ GACTGTGAAGGAACGCTGGT R5′ AATAGGATTAGTCATCACAGGG	58	151	150–268	49	0.497	0.971	AF344657
EJ27.2	$(GAGAA)_{15}$	F5′ AGAGAGACATAACCCTGTGATGA R5′ GGATCATTGGCTCCTCCTATC	60	151	154–267	40	0.550	0.963	AF344660
EJ35	$(TG)_{15}$	F5′ AGTGAGAGGACTCGCAAAGC R5′ CACACGAAGACAGACAAGCAA	60	177	177–270	37	0.746	0.930	AF344658
EJ41.1	$(CACAA)_8$	F5′ TCTACCCCTGGAGGACACAC R5′ ACAGGGGGTTGAGAAAGAGG	55	185	144–192	35	0.746	0.751	AF344659

T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity.

Individual genotypes were determined by polymerase chain reaction (PCR) with radioactive primers. Each PCR reaction totaled 10 μL, containing 100 ng template DNA, 10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton X-100, 0.25 mM dNTP, 1.5 mM Mg^{+2}, 0.25 U *Taq* DNA polymerase (Promega), and 0.3 μM of each primer, with one end-labelled with [γ^{33}P]-ATP. Amplification was carried out by the thermal profile: 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, optimal annealing temperature (Table 1) for 1 min, 72 °C for 1 min and final extension step at 72 °C for 10 min. PCR products were run on regular denaturing 6% polyacrylamide sequencing gel. Sequence of pUC18 was used as size marker to determine the allele sizes. The allelic PCR products differed in multiples of their repeat motifs. The genotypes were scored by two individuals independently and only consistent results were included for analyses.

Six microsatellites were found to be highly polymorphic among adult anchovies collected from waters near Taiwan (Table 1). The number of alleles per locus ranged from 35 to 53, and the observed and expected heterozygosity ranged from 0.438 to 0.746, and from 0.751 to 0.971, respectively (Table 1). The observed genotypes were deviated from Hardy–Weinberg expectation at five out of the six loci ($P < 0.05$) resulting from heterozygote deficiency. Should these fishes be representatives of different stocks, the deficiency may be due to the combination of samples from the different localities of their captures (therefore different stocks). However, the presence of null alleles could also cause the heterozygote deficiency. The large numbers of alleles is notable and may reflect the fact of the large effective population sizes of the species. These microsatellites can be used as genetic markers for identifying stocks to establish regulations in fishing management.

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